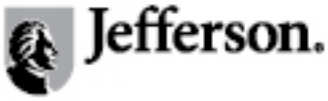

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25(OH)D_3 and Cardiovascular Risk Factors in Female Nonhuman Primates

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Abstract

Objective: To determine if interindividual differences in plasma concentrations of 25-hydroxyvitamin D_3 (25(OH)D_3) have pathophysiologic significance, we evaluated a cohort of female monkeys, seeking to identify associations with clinically relevant cardiovascular risk factors, including age, abdominal obesity (waist circumference), and high-density lipoprotein cholesterol (HDL-C).

Methods: One hundred fifty-five female vervet monkeys (*Chlorocebus aethiops sabaeus*) aged 3–25 years consumed a typical western diet for 7–8 weeks that provided a woman's equivalent of approximately 1000 IU/day of vitamin D_3. Measurements of vitamin D_3 and HDL-C concentrations, as well as waist circumference, were obtained.

Results: Among young monkeys (aged 3–5 years), compared to older monkeys (aged 16–25 years), the mean plasma 25(OH)D_3 concentrations were 82.3 ± 3.2 ng/mL and 58.6 ± 2.9 ng/mL ($p < 0.0001$), respectively. Plasma 25(OH)D_3 concentrations had a range of 19.6–142.0 ng/mL (mean ± standard error [SE] 66.4 ± 1.7 ng/mL). 25(OH)D_3 concentrations were inversely associated with age ($p < 0.0001$) and waist circumference ($p = 0.016$) and were positively correlated with HDL-C ($p = 0.01$). However, when statistically controlling for age, none of these relationships remained significant.

Conclusions: Higher plasma concentrations of 25(OH)D_3 were associated with more favorable cardiovascular risk factors, with inverse associations observed between 25(OH)D_3 and abdominal obesity, HDL-C, and age. These associations were no longer significant when controlling for age.

Introduction

It has been well established that vitamin D is an essential nutrient associated with calcium absorption and bone health. The link between vitamin D and extraskeletal benefits has not been well established, and it is a frequently debated topic. There is mounting evidence, however, associating low vitamin D plasma concentrations or dietary intake with cardiometabolic disorders, such as cardiovascular disease (CVD)[1,2] and type 2 diabetes.[3,4] In addition, evidence has linked vitamin D deficiency to risk factors for the development of CVD,[5] including age,[6] obesity,[7] hypercholesterolemia,[8,9] elevated triglycerides,[10] and diabetes mellitus.[11] There is also evidence that women have lower vitamin D concentrations than men.[12] Retrospective and cohort studies have suggested an association between low plasma concentrations of vitamin D and an increased risk for coronary artery atherosclerosis (CAA).[13,14] Furthermore, the results of recent studies indicate a deficiency in vitamin D may be a risk factor for the metabolic syndrome, which is a major risk factor for development of CAA.[15–17] The National Cholesterol Education Program's Adult Treatment Panel III clinically defines the metabolic syndrome as having three or more of the following characteristics: abdominal obesity, elevated triglycerides, decreased high-density lipoprotein cholesterol (HDL-C), hypertension, and elevated fasting glucose.[18] The metabolic syndrome is a highly prevalent condition among adults living in the United States,[19] making it an important health issue.

The Institute of Medicine's (IOM) latest dietary reference intakes (DRI) for vitamin D are 600 IU for individuals age ≤70 and 800 IU for those >age 70.[20] Despite data suggesting a cardiovascular benefit with adequate vitamin D concentrations,[1,21] the IOM committee reviewed vitamin D's role in extraskeletal health outcomes and made an assessment that the current evidence only supports vitamin D's beneficial role in bone health. The report indicates there is a need for

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additional targeted research, however, and the committee has not ruled out the possibility of suggesting higher intakes in the future if the necessary evidence becomes available.[22]

When analyzing data from retrospective, observational, and cohort studies, confounding variables may exist that can be difficult to control. Retrospective populations, for example, often have risk factors present in the population that can lead to lower vitamin D levels and a greater risk of coronary heart disease (CHD). For these reasons, studies that use nonbiased or less-biased populations would be extremely beneficial and important to further our knowledge about the link between vitamin D and extraskeletal health outcomes.

Therefore, the objective of this study was to investigate whether abdominal obesity, total plasma cholesterol (TPC), HDL-C, and age are associated with plasma concentrations of 25-hydroxyvitamin D_3 (25(OH)D_3) in a cohort of female vervet/African green monkeys. In this study, the use of a nonhuman primate model allows for tight control of dietary intake and environmental conditions, factors that frequently lead to confounding variables in human studies.

Materials and Methods

Subjects

The subjects in this study were 155 nonpregnant, female vervet/African green monkeys (*Chlorocebus aethiops sabaeus*) from the Vervet Research Colony (VRC) of the Wake Forest University Primate Center (WFPC). The VRC is a multigenerational, pedigreed, and genotyped colony that was founded in 1975 with 57 animals imported from the islands of St. Kitts and Nevis. The VRC has been a closed colony since the mid-1980s, with no new animals imported since that time. In early 2008, the VRC was transferred from its original location in California to the WFPC. At the time of the study, the colony consisted of 486 animals in their second to eighth generation. All animals were colony born and were of known age.

Housing

All subjects were housed in 16 social groups at the WFPC. Each of the 16 housing pens consisted of a large outdoor area (~1200 square feet) and a divided indoor area (300 square feet). All pens were fitted with elevated perches, platforms, and climbing structures. Each of the 16 social groups contained one or two adult males along with varying numbers of adult females and immature offspring. In general, females remained in their natal groups along with their mothers and sisters. Males were removed at 4 years old to prevent inbreeding, and adult males were replaced every 3–5 years. Population size was controlled by vasectomizing males and periodic culling. At the time of the study, each of the 16 social groups housed between 11 and 49 animals (mean ± standard deviation [SD]: 30.4 ± 11.7).

Diet

Before the study, all monkeys were fed a similar diet that contained 6.6 IU/g of added vitamin D_3, or the human equivalent of 4000 IU/day. Then, as part of this study, all monkeys were fed a challenge diet. This challenge diet was formulated to mimic the typical American diet deriving 37% of calories from fat, 18% from protein sources (mostly animal), and 45% from carbohydrates and containing 0.18 mg/Cal of cholesterol. The diet contained 3.0 IU/g of added vitamin D_3, or the human equivalent of 1000 IU/day. A comparison of the two diets is shown in Table 1. Throughout the study, animal diets were supplemented with fresh fruits and vegetables three times per week. All animals had *ad libitum* access to food and water and opportunities to exercise.

Experimental procedures

Animals were sampled 7–8 weeks after they began consuming the challenge diet. One or two entire social groups were sampled per day, and the entire sampling period took place over 2–3 weeks. Initiation of the challenge diet was staggered so that a similar amount of time elapsed between the start of the diet change and the collection of samples. On each sampling day, animals were fasted overnight, captured, and anesthetized with an intramuscular injection of 15 mg/kg ketamine HCl. Animals were weighed, morphometric measures were taken, and blood was collected via femoral venipuncture using 6 mL ethylenediaminetetraacetic acid (EDTA) Vacutainers and 3.5-mL serum-separating tubes (SST) tubes. The EDTA blood samples were put on wet ice immediately after collection, and the SST tubes were kept at ambient temperature. After sampling was completed for the day, the samples were transferred to the laboratory and centrifuged for 25 minutes at 1000*g*. Aliquots of plasma were taken for lipid (600 μL) and glycemic (400 μL) assays from the EDTA tubes, and 500 μL aliquots of serum were taken from the SST tubes for vitamin D assays. All samples were frozen at −20°C after initial processing and then transferred to a −80°C freezer until assays were performed.

Morphometric measurements included body weight (BW), waist circumference (WC), crown-rump length (CR), and measurement of the suprasternal notch to the pubic symphysis distance (SNPS). WC was measured while animals were in the supine position using a flexible tape measure that was placed around the monkey's abdomen at the level of the umbilicus. The two length measurements were recorded using digital calipers while the animals were placed on their side (CR) or supine (SNPS). Weight was measured in kilograms (kg), and all other measurements were recorded in centimeters (cm). Separate body mass indices (BMI) were calculated from each length measurement (kg/cm^2). Interrater reliabilities were maintained at >95% agreement.

Pregnancy status of adult females was determined by ultrasound on the date of sampling or if the animal gave birth within 165 days of the sampling date (a time frame that would indicate they were pregnant at the time of sampling). Ultrasound measurements were made by a trained veterinarian

Table 1. Diet Formulations for Standard (Preliminary) Chow Diet and Challenge High-Fat Diet

	Preliminary diet[a]	*Challenge diet*[b]
Fiber (crude)	5%	9%
Calories by protein	18%	18%
Calories by fat	13%	37%
Calories by carbohydrates	69%	45%
Metabolizable energy (kcal/g)	3.22	3.34
Vitamin D_3 (IU/g)	6.6	3.0
Women's equivalent (IU/day)	4000	1000

[a]Chow (Purina LabDiet 5038).
[b]Typical American Diet (Purina LabDiet 5L0P).

(SonoSite 180, Sono-Site Inc.) using previously established protocols in this colony.[23]

Assays

Frozen serum samples (500 μL) were assayed for 25(OH)D (vitamin D_2 and D_3) using HPLC/tandem mass spectrometry at the Reading Hospital and Medical Center. Since 25(OH)D_2 levels were often below the level of detectability, only 25(OH)D_3 data will be presented. Samples were protected from direct sunlight throughout the process. Analyses used an Applied Biosystems/MDS Sciex 3200 QTRAP liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/2) and a Shimadzu Prominence 20A LC System. Sample preparation was based on a liquid-liquid extraction using acetonitrile to precipitate proteins, with reconstitution in methanol.

EDTA samples were assayed for the following variables: total plasma cholesterol (TPC), triglycerides (TG), HDL-C, very low-density lipoprotein cholesterol (VLDL-C), and low-density lipoprotein cholesterol (LDL-C). All of the variables were measured and reported in mg/dL. Lipoprotein cholesterol distributions were determined using size separations of lipoprotein classes via gel filtration chromatography.[24] An aliquot of plasma (containing about 15 μg of cholesterol) diluted 1:1 with cold phosphate-buffered saline (PBS) was applied to a Superose 6 column (GE Healthcare). The column was eluted with 0.9% saline containing 0.01% EDTA and 0.01% sodium azide at a flow rate of 0.4 mL/min using a LaChromElite HPLC system (Hitachi High Technologies), and the column eluate was continuously mixed online with 0.125 mL/min cholesterol reagent (Cholesterol Liquid Reagent Set, Pointe Scientific, Inc.), which was then passed through a 5-mL knitted reaction coil maintained at 37°C. Data readout is proportional to cholesterol concentration in the eluate, and fractions containing VLDL, LDL, and HDL were identified so that the percentage of cholesterol in each could be determined. The cholesterol concentrations in each lipoprotein class were then calculated from a direct measure of cholesterol concentration using an enzymatic colorimetric assay in an aliquot of the starting plasma.

Plasma glucose (GLU, Sigma-Aldrich) and fructosamine (FRUC, Roche Diagnostics) were assayed by enzymatic colorimetric methods. The interassay and intraassay coefficients of variation (CV) % were $<5\%$ for GLU and $<10\%$ for FRUC. Insulin (INS) was determined using ELISA (Mercodia), with interassay and intraassay CV being $<10\%$.

Approvals

All procedures were approved by the Wake Forest Institutional Animal Care and Use Committee (IACUC). Wake Forest is an AAALAC accredited institution, and all animal care procedures followed the NIH Guide.

Statistics

Unless otherwise noted, all results are reported as mean ± standard error of the mean (SE). All statistical analyses were performed using SAS (version 9.1). Associations were measured using Pearson correlations, and mean differences were tested using analysis of variance (ANOVA). F-test results with degrees of freedom (df) are reported in addition to p values, with p values <0.05 considered statistically significant. Associations controlling for relatedness of individuals were performed using Sequential Oligogenic Linkage Analysis Routines (SOLAR).[25]

Results

A total of 238 females were originally sampled, although analyses were restricted to 155 nonpregnant females. Table 2 summarizes the descriptive statistics for the variables measured in this study. These females ranged in age from 3.5 to 24.7 years (10.4 ± 0.4), and BW ranged from 3.3 to 7.7 kg (5.1 ± 0.1). WC ranged between 24.5 and 48.5 cm (33.3 ± 0.3). The distribution of 25(OH)D_3 concentrations is shown in Figure 1. The range of 25(OH)D_3 concentrations was 19.6–142.0 ng/mL, with a mean ± SE of 66.4 ± 1.7.

As shown in Figure 2, plasma 25(OH)D_3 concentrations were inversely associated with age ($r = -0.35$, $p < 0.0001$). As show in Figure 3, among young monkeys (aged 3–5 years), mean plasma 25(OH)D_3 concentrations were 82.3 ± 3.2 ng/mL vs. 58.6 ± 2.9 in older monkeys (aged 16–25 years) ($F(3,151) = 13.79$, $p < 0.0001$). Plasma 25(OH)D_3 concentrations were inversely associated with WC ($r = -0.19$, $p = 0.016$). The

Table 2. Descriptive Statistics of All Variables

Variable	*Name (units)*	n	*Mean*	*SE*
25(OH)D_3	25(OH)D_3 (ng/mL)	155	66.4	1.7
Age	Age (years)	155	10.4	0.4
BW	Body weight (kg)	155	5.1	0.1
WC	Waist circumference (cm)	153	33.3	0.3
CR	Crown-rump length (cm)	153	44.2	0.1
SNPS	Suprasternal notch to pubic symphysis distance (cm)	153	29.5	0.1
BMI (CR)	Body mass index w/CR (kg/cm^2)	153	25.9	0.3
BMI (SNPS)	BMI w/SNPS (kg/cm^2)	153	58.4	0.7
TPC	Total plasma cholesterol (mg/dL)	155	207.6	3.4
HDLC	High-density lipoprotein cholesterol (mg/dL)	155	109.0	2.3
TPC:HDLC	TPC/HDL-C ratio	155	2.0	0.0
VLDLC	Very low density lipoprotein cholesterol (mg/dL)	155	2.0	0.1
LDLC	Low-density lipoprotein cholesterol (mg/dL)	155	96.6	2.4
VLDLC + LDLC	VLDL-C + LDL-C	155	98.6	2.4
TG	Triglycerides (mg/dL)	155	36.9	1.5
GLU	Glucose (mg/dL)	155	65.5	1.5
FRUC	Fructosamine (umol/L)	155	154.2	2.5
INS	Insulin (mU/L)	155	14.1	1.4

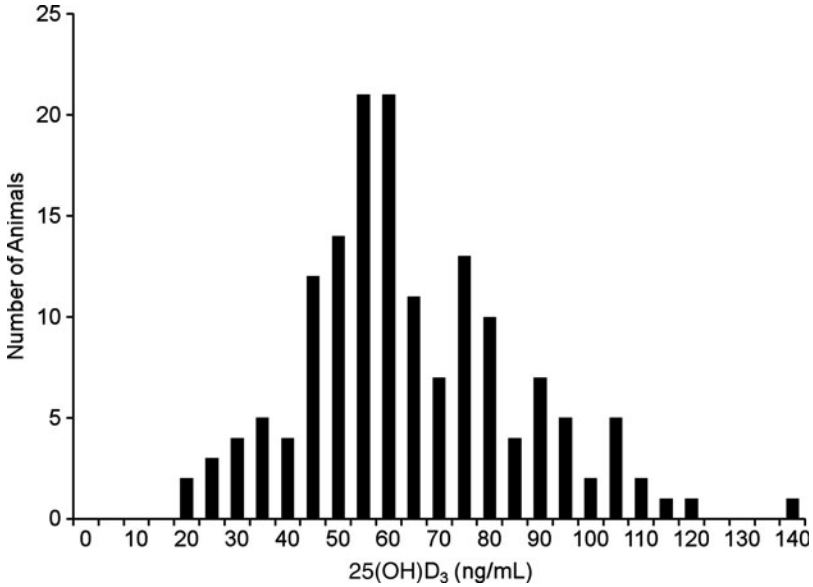

FIG. 1. Histogram of vitamin D_3 concentrations.

females in the lowest quartile (Q1) of 25(OH)D_3 concentrations had WC of 34.2 ± 0.6 cm vs. the highest quartile (Q4) with WC of 31.5 ± 0.7 cm ($F(3,149) = 32.48$, $p < 0.05$) (Q2 = 33.4 ± 0.6 cm, Q3 = 34.0 ± 0.6 cm). Plasma 25(OH)D_3 concentrations were positively correlated with HDL-C ($r = 0.20$, $p = 0.01$) (Figure 4). Thus, the lowest quartile of 25(OH)D_3 had mean HDL-C concentrations that were 14.9 mg/dL less than the highest quartile ($F(3,151) = 1.88$, $p = 0.14$) (Q1 = 102.8 ± 4.3 mg/dL, Q2 = 106.7 ± 4.1 mg/dL, Q3 = 214.5 ± 7.5 mg/dL, Q4 = 117.7 ± 4.6 mg/dL).

Table 3 shows the correlations between the different cardiometabolic risk factors (morphometric, lipid, and glycemic) and 25(OH)D_3 concentrations, age, and 25(OH)D_3 concentrations with age as a covariate. Whereas a number of variables were correlated with 25(OH)D_3 concentrations, many of these variables were also correlated with age. When controlling for age statistically, only the positive relationship between 25(OH)D_3 and total cholesterol remained significant ($r = 0.19$, $p = 0.0204$).

Because all the animals in this study were part of one large pedigree, the assumptions of independence of observations were violated. Separate analyses of the correlations were performed with SOLAR, taking into account the relatedness of individuals. None of the correlations were significantly altered by the inclusion of relatedness as a factor. It should also be noted that calcium concentrations from 152 of the 155 animals, measured as part of annual veterinary health screening, were positively (though not significantly) correlated with 25(OH)D_3 concentrations.

Discussion

Using these nonhuman primates as surrogates for aging women, we have shown that lower concentrations of

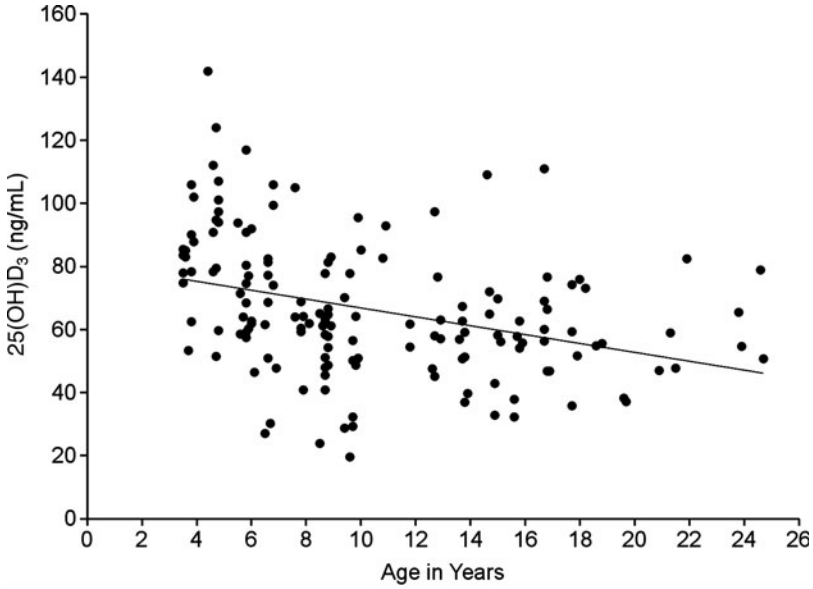

FIG. 2. Scatterplot of 25(OH)D_3 concentrations (ng/mL), by age in years ($r = -0.35$, $p < 0.0001$).

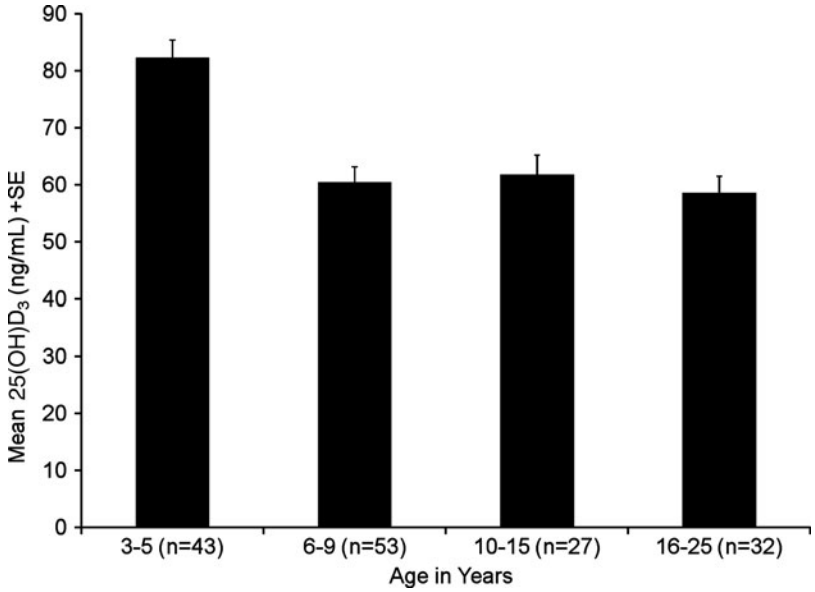

FIG. 3. Mean + standard error (SE) of $25(OH)D_3$ concentrations by age category.

$25(OH)D_3$ are significantly associated with increasing age, higher abdominal obesity, and decreased HDL-C and TPC. Conversely, higher plasma concentrations of $25(OH)D_3$ are associated with cardiometabolic parameters that support a potential mechanism for cardioprotection. Our findings are consistent with studies that have found significant associations between vitamin D and abdominal obesity,[7] age,[6] and HDL-C[8,9] in human populations. When statistically controlling for age, however, only the relationship between $25(OH)D_3$ and TPC remained significant. Although the direct association between $25(OH)D_3$ and TPC may appear to be unexpected, we believe our study model provides a logical explanation. More specifically, TPC and HDL-C tend to covary in vervets.[26] It is likely, therefore, that the TPC effect is being driven by HDL-C in this species and, hence, presents a slight variation from the human model and one that should not divert our focus from the other significant and relevant findings. Previously, we have shown a significant association between low plasma concentrations of $25(OH)D_3$ and lower HDL-C in a cohort of female cynomolgus monkeys (*Macaca fascicularis*),[9] an effect that is consistent with the positive correlation between $25(OH)D_3$ and HDL-C in the present report. Numerous reports have shown that vitamin D is associated inversely with age[6,27] and obesity.[1,7,28] However, less well understood is whether the association is the result of old age and obesity, leading to lifestyles that cause lower plasma concentrations of vitamin D. For example, persons of older age can reside in assisted living facilities and, therefore, have less sun exposure and less plasma vitamin D concentrations. In addition, obese individuals may have less exposure to

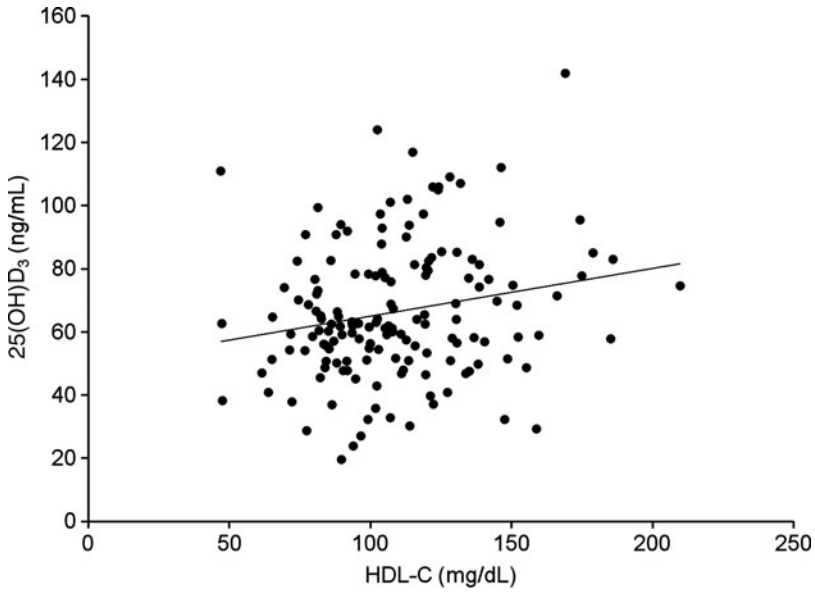

FIG. 4. Scatterplot of $25(OH)D_3$ concentrations (ng/dL) by high-density lipoprotein cholesterol (HDL-C) concentrations (mg/dL) ($r = 0.20$, $p = 0.01$).

Table 3. Correlations Between Cardiovascular Risk Factors and a) $25(OH)D_3$, b) Age, c) $25(OH)D_3$ with Age as Covariate (n = 153–155)

	Pearson correlation with 25(OH)D_3		*Pearson correlation with age*		*Partial correlation with 25(OH)D_3 (age as covariate)*	
Variable	r	p	r	p	r	p
BW	−0.13	0.10	**0.27**	**<0.001**	−0.04	0.60
WC	**−0.19**	**0.02**	**0.39**	**<0.0001**	−0.07	0.41
CR	0.06	0.45	0.05	0.56	0.08	0.31
SNPS	0.04	0.61	0.10	0.21	0.08	0.31
BMI (CR)	**−0.17**	**0.04**	**0.28**	**<0.001**	−0.08	0.35
BMI (SNPS)	−0.16	0.05	**0.22**	**<0.01**	−0.09	0.28
TPC	**0.22**	**0.01**	−0.14	0.08	**0.19**	**0.02**
HDLC	**0.20**	**0.01**	**−0.20**	0.01	0.14	0.07
TPC:HDLC	0.01	0.88	0.13	0.11	0.06	0.45
VLDLC	0.02	0.80	0.16	0.05	0.08	0.31
LDLC	0.12	0.14	−0.01	0.87	0.12	0.13
VLDLC + LDLC	0.12	0.14	−0.01	0.94	0.12	0.13
TG	0.03	0.74	**0.26**	**0.001**	0.13	0.10
GLU	−0.04	0.60	**0.20**	**0.02**	0.03	0.73
FRUC	−0.03	0.74	0.10	0.21	0.01	0.91
INS	0.07	0.41	0.04	0.62	0.09	0.29

Bold face type indicates significant findings.

ultraviolet light because of decreased outdoor physical activity. It has been suggested that because of the fat-soluble nature of vitamin D, the major storage form of vitamin D, $25(OH)D_3$, can be sequestered in adipose tissue, causing decreased plasma concentrations of vitamin D in obese individuals.[29]

Vitamin D deficiency is a widespread health concern. The Third National Health and Nutrition Examination Survey reports vitamin D deficiency may be present in 25%–57% of American adults.[30] Less well understood, however, is whether the large individual differences in plasma concentrations of vitamin D have any extraskeletal pathophysiologic significance. As vitamin D supplementation is becoming a popular method used by clinicians to raise vitamin D concentrations, it was very interesting to see $25(OH)D_3$ concentrations ranging from 19.6 to 142.0 ng/mL in this cohort. Recall, despite this wide variation, that each monkey was fed a diet with identical doses of oral vitamin D (1000 IU/day) along with similar housing conditions and sun exposure. This finding has been observed in human populations as well, where studies suggest between 18% and 53% of individuals will respond to vitamin D supplementation.[31,32] The outdoor housing and supplementation of the diet also help explain why very few of the animals in this study showed $25(OH)D_3$ concentrations < 40 ng/dL, levels that would be considered low in a typical human population.

Future studies on this topic would be beneficial. A prospective analysis that examines the effects of vitamin D supplementation on abdominal obesity, TPC, HDL-C, and the metabolic syndrome would be clinically beneficial. In addition, a study that seeks to explain the mechanism for the link among vitamin D, TPC, and HDL-C would further our knowledge on vitamin D and its role in CVD. Further investigations explaining why such large differences in vitamin D concentrations are observed after supplementing with 1000 IU/day and what causes an individual not to respond to supplementation would have major clinical significance. Finally, a randomized controlled trial using vitamin D and designed to test for CVD as the primary outcome would be beneficial, as these types of analyses are nearly nonexistent.

Conclusions

The clinical relevance of our findings is important. This report suggests an explanation for why individuals with low plasma concentrations of vitamin D may be at an increased risk for CHD. In our sample, female vervet/African green monkeys with lower plasma $25(OH)D_3$ concentrations had significantly lower HDL-C levels and greater abdominal obesity, both of which are well-recognized risk factors for the development of CHD. In addition, we observed a significant inverse relationship between age and plasma concentration of $25(OH)D_3$. It should be noted that when age was used as a covariate, many of the associations between $25(OH)D_3$ and other CVD risk factors were no longer significant. The study was done in such a way that the entire cohort had the same housing conditions and was fed the same diet, thus reducing variability in sun exposure and vitamin D intake. This approach allowed for finer control of variables that confound similar studies of human subjects.

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Disclosure Statement

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